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SHORT COMMUNICATION

pH-Responsive PEGylated nanogels as targetable and low invasive endosomolytic agents to induce the enhanced transfection efficiency of nonviral gene vectors

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Abstract A pH-responsive PEGylated nanogel was successfully prepared by means of emulsion copolymerization of 2-(*N*,*N*-diethylamino)ethyl methacrylate (AMA) with heterobifunctional poly(ethylene glycol) (PEG) bearing a 4-vinylbenzyl group at the α-end and a lactose moiety at the ω-end in the presence of potassium persulfate and ethyleneglycol dimethacrylate as a cross-linker. Polyplex micelle composed of PEG-*block*-poly(L-lysine) copolymer and plasmid DNA (PEG-*b*-PLL/pDNA) exhibited a far more efficient transfection ability in the presence of lac-nanogel-8k-1.0% (PEG, M_n=8000; cross-linking density, 1.0%) than the PEG-*b*-PLL/pDNA polyplex micelle alone (in the absence of lac-nanogel-8k-1.0%), suggesting that an appreciable fraction of lac-nanogel-8k-1.0% along with the PEG-*b*-PLL/pDNA polyplex micelle is taken up into the HuH-7 cells through the asialoglycoprotein receptor-mediated endocytosis process mediated by the cluster of a large number of lactose moieties on the surface of lac-nanogel-8k-1.0%, followed by the effective disruption of the endosome by the buffer effect of the unprotonated PAMA core in lac-nanogel-8k-1.0%.

Keywords Core-shell type nanogel · pH-sensitive volume phase transition · Endosomolytic agent · Gene vector · PEG-tethering surface

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Introduction

Nonviral gene delivery systems have recently received increased attention in the field of gene therapy in vivo and ex vivo because of concerns over safety issues related to viral vectors, including immunogenicity, oncogenicity, and potential virus recombination [1–3]. Most of the nonviral vectors developed so far, however, have shown a low transfection efficiency compared to viral vectors because the latter have evolved a multifunctionality, which overcomes one of the critical barrier to efficient gene delivery by enhancing transport to the cytoplasm from the endosomal compartment. Recently, a new class of nonviral gene vectors has been developed based on the supramolecular assembly between plasmid DNA (pDNA) and poly(ethylene glycol) (PEG)-*block*-polyamine copolymers (polyplex micelles) [4–10]. Due to the highly dense PEG shell surrounding the polyion complex (PIC) core, the polyplex

micelles with a size of less than 100 nm exhibited excellent solubility in aqueous media, low cytotoxicity, high tolerability toward nuclease degradation, and minimal interaction with biological components, including proteins and cells, compared to conventional polyplex and lipoplex systems.

Nevertheless, the presence of 100 μM hydroxychloroquine (HCQ) as an endosomolytic agent has so far been required to achieve a high transfection efficiency [2, 11], indicating that endosomal escape should be the most critical barrier to intracellular gene delivery by polyplex micelles [12, 13]. To devise polyplex micelles with a function to escape from the endosome where the pH is 1.4~2.4 units lower than the physiological pH of 7.4 [14–17], poly (ethylenimine) (PEI) derivatives are of interest as an alternative way to accomplish endosomal escape by taking advantage of their substantially lowered value of apparent pK_a (~5.5; "buffer or proton-sponge effect") [18]. However, the buffer effect of the PEI segment occurs only when an excess of amino groups with respect to DNA phosphate groups (high N/P ratio) is present in the system, where a considerable amount of the amino groups in PEI is in free-base form. This fact strongly suggests that free PEI, which is not complexed with pDNA, is likely to play a crucial role in the buffer effect [19, 20]. In addition, both HCQ and free PEI tend to show high toxicity and nonspecific disposition in the body after intravenous injection, viz., the use of HCQ and PEI for the gene therapy under in vitro and ex vivo conditions is still controversial. A major key to the success of nonviral gene delivery system is believed to be the development of targetable and low invasive endosomolytic agents, which can achieve the low cytotoxicity and modulated disposition in the body as well as the smooth accumulation into the target cell.

Worth noting in this regard is a new class of pH-responsive PEGylated nanogels constructed from a cross-linked pH-sensitive polyamine core and tethered PEG chains bearing a carboxylic acid group as a platform moiety to install ligand molecules [21]. The pH-responsive nanogels showed excellent stability under physiological conditions and significant volume phase transition (swelling) in response to endosomal pH (pH>7.5, diameter ca. 80 nm; pH<6.5, diameter ca. 150 nm; see Fig. S1) due to the protonation of the cross-linked polyamine core surrounded by the tethered PEG chains, indicating that the cross-linked polyamine core of the nanogels acts as a buffering moiety for facilitated endosomal escape. A unique finding, which we would like to communicate in this paper, is the remarkably enhanced transfection efficiency without cytotoxicity in cultured hepatoma cells through the PEGylated polyplex micelle composed of PEG-*block*-poly (L-lysine) copolymer (PEG-*b*-PLL: $M_{n\ PEG}$=12,000, $M_{n\ PLL}$=11,600) and pDNA along with pH-responsive lactosylated nanogel (Fig. 1); thus, the pH-responsive lactosylated nanogel is promising as a targetable and

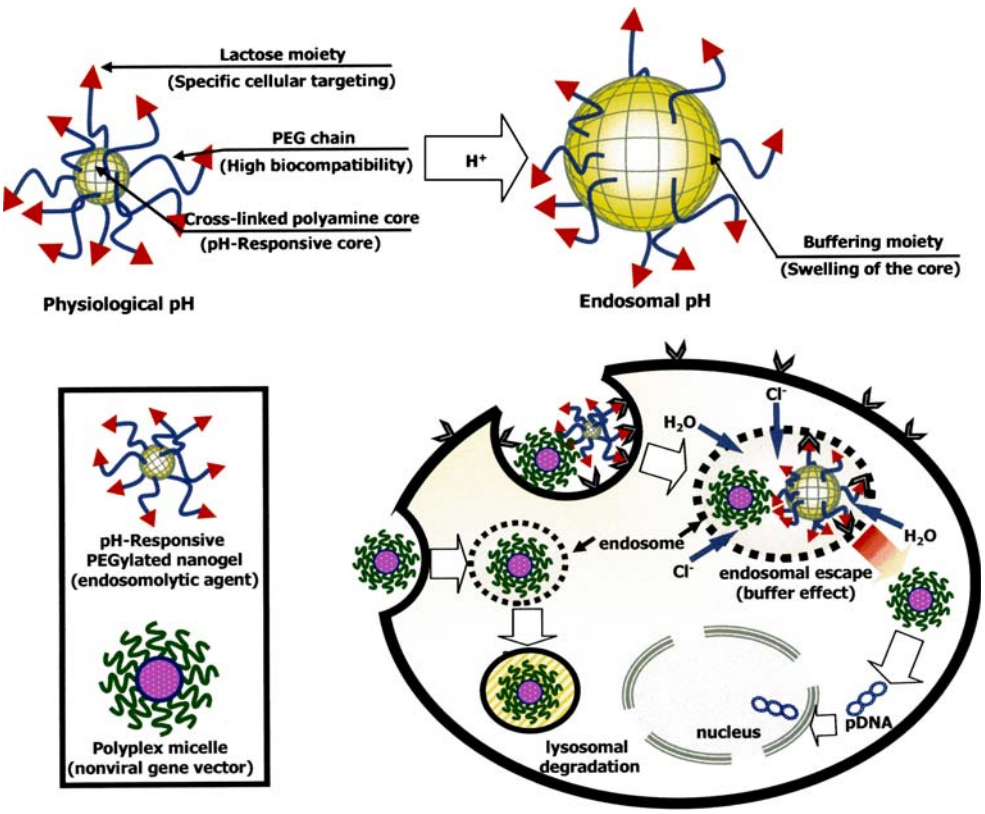

Fig. 1 Schematic illustration of the pH-responsive PEGylated nanogel and endosomal escape mechanism

biocompatible endosomolytic agent for nonviral gene delivery systems.

Experimental

General Ethylene glycol dimethacrylate (EGDMA; Wako) and 2-(*N*,*N*-diethylamino)ethyl methacrylate (AMA, Wako) were distilled over CaH_2 under reduced pressure. Potassium persulfate (KPS; Wako) was purified by recrystallization from water and then dried in vacuo. Asialofetuine (ASF) and HCQ were purchased from Sigma and Acros Organics, respectively. Water was purified using the Milli-Q system (Millipore). Plasmid DNA (pDNA) encoding firefly luciferase (pGL3-Luc, Promega; 5,256 bpa) was amplified using EndoFree™ Plasmid Maxi or Mega Kits (Qiagen). The DNA concentration was determined by reading the absorbance at 260 nm. Dynamic light scattering (DLS) measurements were carried out using a light-scattering spectrometer (DLS-7000, Otsuka Electronics. Japan) equipped with a vertically polarized incident beam at 488 nm supplied by an argon ion laser at scattering angles of 90°. Lasser–Doppler electrophoresis measurements of the PEGylated nanogels were carried out in 10 mM $NaCl_{aq}$ (ELS-600, Photal, Otsuka Electronics).

Synthesis of CH_2=CH–Ph–PEG–lactose and preparation of nanogels Heterobifunctional α-vinylbenzyl-ω-carboxyl-poly(ethylene glycol) [CH_2=CH–Ph–PEG–COOH; molecular weights (MW): 2k and 8k] macromonomers were synthesized in accordance with our previous report [21]. To a solution of potassium 4-vinylbenzyl alcoholate (0.5 mmol) in tetrahydrofolate (THF; 20 ml), 22.7 mmol (1.1 ml) of ethylene oxide were added under an argon atmosphere. After the reaction mixture was stirred at room temperature for 2 days, 2.5 mmol (3.4 ml, 0.725 mol/l in THF solution) of succinic anhydride was added to introduce a carboxylate group at the ω-end. After the purification of CH_2=CH–Ph–PEG–COOH, lactose was installed at the carboxylic acid group of CH_2=CH–Ph–PEG–COOH through an activated ester method, viz., 8.0 mg of CH_2=CH–Ph–PEG–COOH (MW: 8k, 10 μmol) were reacted with 22 mg of *p*-aminophenyl-β-D-lactopyranoside (50 μmol) in the presence of 250 μmol of *N*-hydroxysuccinimide (NHS) and 1.25 mmol of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride (EDC; NHS/EDC ratio of 1:5) in 25 mM 2-morpholinoethanesulfonic acid (MES) buffer (10 ml), pH 6.5. The reaction mixture was stirred at room temperature for 24 h. The CH_2=CH–Ph–PEG–lactose was precipitated in cooled 2-propanol (twice) and dialyzed (MWCO: 3,500) against distilled water for 2 days. The CH_2=CH–Ph–PEG–lactose was finally freeze-dried from water to quantitatively obtain the white powder. We used a typical procedure for the preparation of the pH-responsive nanogels possessing a lactose moiety at the PEG chains, as follows: after 65 mg (8.1 μmol) of the obtained CH_2=CH–Ph–PEG–lactose (8k) were loaded into the reactor, the vacuum and argon purging cycles were repeated three times, followed by the successive addition of deionized/distilled water (1.5 ml), 130 μg of 2-(*N*,*N*-diethylamino) ethyl methacrylate (AMA, 141 μl, 700 μmol) and 1.5 μl of ethyleneglycol dimethacrylate (EGDMA, 7.1 μmol). Emulsion copolymerization was initiated with the addition of 1 ml of aqueous KPS (7.2 mM). The mixture was allowed to react at room temperature for 24 h with stirring. The nanogel was purified by dialysis against distilled water for 2 days.

Cytotoxicity assay Human hepatocarcinoma cells (HuH-7) were seeded onto 96-well plates at a seeding density of 8×10^3 cells/well. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1.0% penicillin–streptomycin for 24 h at 37 °C in a humidified atmosphere under 5% CO_2. Then, the culture medium was replaced by 90 μl of fresh medium containing serum and antibiotic, followed by the addition of 10 μl of the nanogels at various concentrations. After 24 h, 100 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT assay, cell counting kit; Dojindo) stock solution in culture medium were added to each well. After an additional 4 h of incubation, the viability of the cells in each well was measured following the protocol provided by the manufacturer. The results are expressed as means±SEM, $n=8$.

Transfection study To a pDNA (pGL3-Luc, Promega; 5,256 bps) solution in 10 mM Tris–HCl buffer (pH 7.4), PEG-*block*-poly(L-lysine) (PEG-*b*-PLL: $M_{n\ PEG}=12{,}000$, $M_{n\ PLL}=11{,}600$), the block copolymer solution in the same buffer, was added at the charge ratio of lysine units vs nucleotide equals 2 (N/P=2) to prepare the polyplex micelle. The HuH-7 cells were seeded in 24-well culture plates at a seeding density of 2.5×10^4 cells/well. After a 24-h incubation in a medium containing 10% FBS, the cells were rinsed, and then 250 μl of the culture medium containing 100 μg/ml of lac-nanogel-8k along with the PEG-*b*-PLL/pDNA polyplex micelle solution (25 μl/well; pDNA concentration, 30 μg/ml) were added to each well. After 24 h, the medium was removed and then replaced with the culture medium. The luciferase gene expression was measured after a further 24 h of culturing. For the competitive inhibition assay of lac-nanogel-8k to the asialoglycoprotein (ASGP) receptor on the HuH-7 cells, a medium containing 1.5 mg/ml of ASF was added to the culture system 30 min before the transfection study. The cells were lysed, and the luciferase activity of the lysate

was monitored with a Luciferase Assay Kit (Promega) and luminometer (Lumat LB 9507, Berthold). The results were expressed as light units per milligram of cell protein determined by a micro BCA assay kit (Pierce).

Results and discussion

Heterobifunctional PEGs bearing 4-vinylbenzyl group at the α-end and a carboxylic acid at the ω-end (CH_2=CH–Ph–PEG–COOH, M_n=1,800 and 8,000) were synthesized, in accordance with our literature [21]. The introduction of a lactose group to the carboxylic acid end of CH_2=CH–Ph–PEG–COOH (M_n=8,000) was performed by the reaction with *p*-aminophenyl-β-D-lactopyranoside in the presence of an excess amount of NHS and EDC in 25 mM MES buffer, pH 6.5, at room temperature for 24 h. Based on the hydrogen nuclear magnetic resonance (^{1}H NMR) spectrum of CH_2=CH–Ph–PEG–lactose (Fig. 2), the lactose molecules were almost quantitatively introduced to the PEG chain end as determined by the integral ratio between the ester metylene protons (4.1 ppm, 2H, PEG–CH_2CH_2–O–CO–CH_2–) and the phenyl protons of the lactose moiety (6.9 ppm, 2H, –CO–NH–Ph–lactose). Lactosylated and nonlactosylated nanogels were prepared at room temperature by means of the emulsion polymerization of 2-(*N,N*-diethylamino)ethyl methacrylate (AMA) with CH_2=CH–Ph–PEG–lactose (M_n=8,000) or CH_2=CH–Ph–PEG–COOH (M_n=1,800 or 8,000) in the presence of KPS and EGDMA (0.1 or 1 mol%) as a cross-linker because the tertiary amino groups in the AMA monomer and KPS spontaneously forms a redox complex (initiator) at room temperature through the electron transfer from AMA to KPS. The characterizations of the obtained nanogels with a unimodal size distribution (μ_2/Γ^2<0.15) are summarized in Table 1.

An MTT assay was done using HuH-7 cells (human hepatoma cells) possessing ASGP receptors, which recognize compounds bearing terminal galactose moieties [22, 23], to evaluate the cytotoxicity of the obtained nanogels, as shown in Fig. 3. Nanogel-2k-1.0% with short PEG chains (M_n=1,800) and nanogel-8k-0.1% with low cross-linking density showed high cytotoxicity in a dose-dependent manner, despite possessing PEG chains surrounding the cross-linked PAMA core: The 50% cytotoxicity concentrations were determined to be ca. 10 and 6 μg/ml, respectively. In addition, the viability of cells treated with HCQ was less than 50% at 100 μM (=31.4 μg/ml). In sharp contrast, nanogel-8k-1.0% and lac-nanogel-8k-1.0% with long PEG chains (M_n=8,000) and moderate cross-linking density (1.0%) showed low cytotoxicity even at 100 μg/ml (>70% cell viability). These results suggest that the cytotoxicity of the nanogels obviously depended on the chain length of the PEG and the cross-linking density of the PAMA core. The observed high cytotoxicity of nanogel-2k-1.0% and nanogel-8k-0.1% is most likely due to partial exposure of the PAMA core to the outside and/or the existence of dangling PAMA chains, as the zeta potential of the fully protonated nanogel-2k-1.0% and nanogel-8k-0.1% at pH 5 was found to be +32.3 and +33.1 mV, respectively. On the contrary, nanogel-8k-1.0% and lac-nanogel-8k-1.0% with longer PEG

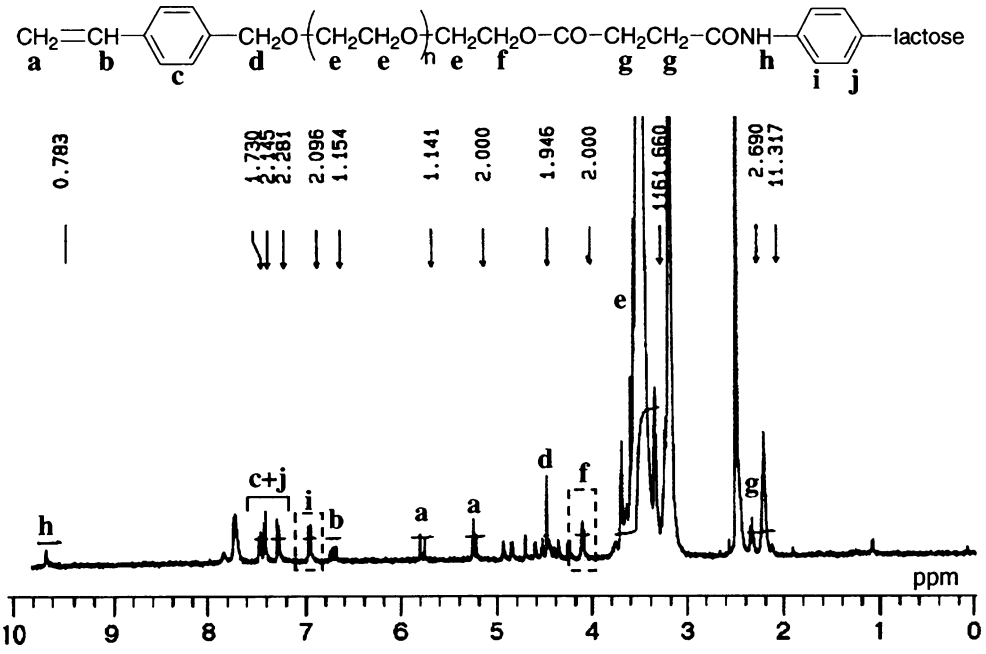

Fig. 2 ^{1}H NMR spectrum of the CH_2=CH–Ph–PEG–lactose in DMSO-d_6 at 55 °C (peaks at 2.5 and 3.2 ppm are attributed to the DMSO and water, respectively)

Table 1 The nanogel samples used in this study

Samples	M_n(PEG)[a]	Cross-linker[b] (mol %)	Particle size[c] (nm)	
			pH 6	pH 8
Nanogel-2k-1.0%	1,800	1.0	128.9	72.0
Nanogel-8k-0.1%	8,000	0.1	320.0	89.3
Nanogel-8k-1.0%	8,000	1.0	152.7	84.4
Lac-nanogel-8k-1.0%	8,000	1.0	160.8	84.7

[a] Determined by SEC chromatography
[b] Feed molar ratio of cross-linking agents: EGDMA
[c] Determined by DLS analysis

chains (M_n=8,000) and higher cross-linking density (1.0%) showed the effective compartmentalization of the cross-linked PAMA core surrounded by long PEG chains to prevent both exposure of the PAMA core to the outside and the formation of dangling PAMA chains (fully protonated nanogel-8k-1.0%, ζ=+14.0 mV), leading to the low cytotoxicity of these nanogels.

To estimate the endosomolytic ability of the nanogels, a transfection study of the PEG-*b*-PLL/pDNA polyplex micelle was carried out in the presence and absence of lac-nanogel-8k-1.0% (100 μg/ml) using HuH-7 cells, although there is no interaction between the polyplex micelle and nanogel due to the existence of the tethered PEG chains (PEG shell). PEG-*b*-PLL/pDNA polyplex micelles were prepared at N/P=2, where the highest transfection efficiency and complete DNA condensation were observed in our previous study [5]. As can be seen in Fig. 4, the PEG-*b*-PLL/pDNA polyplex micelle alone

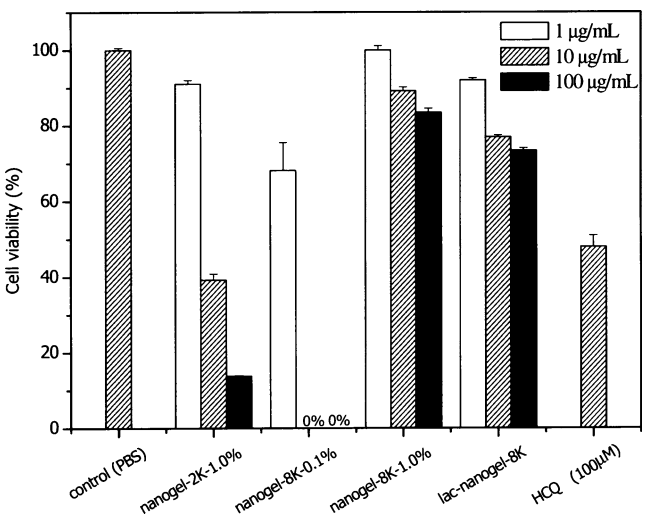

Fig. 3 Cytotoxicity of various types of the nanogels and HCQ. The relative viability of HuH-7 cells is expressed as functions of nanogel concentrations: 1 μg/ml (*open bars*), 10 μg/ml (*gray bars*), and 100 μg/ml (*filled bars*). The results are expressed as means±SEM, n=8

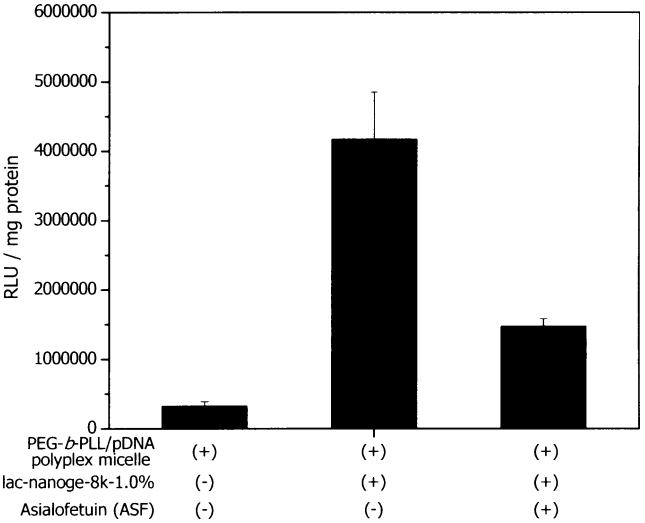

Fig. 4 Efficiency of transfection into HuH-7 cells of the PEG-*b*-PLL/pDNA polyplex micelles prepared at N/P=2. The lac-nanogel-8k-1.0% (100 μg/ml) and ASF (1.5 mg/ml) were used. The results are expressed as means±SEM, n=4

(without lac-nanogel-8k-1.0%) showed only limited transfection efficiency without any cytotoxicity due to the lack of the endosomal escape function, leading to the lysosomal degradation of the pDNA. In sharp contrast, the PEG-*b*-PLL/pDNA polyplex micelle with lac-nanogel-8k-1.0% achieved a significant increase in transfection efficiency compared with the PEG-*b*-PLL/pDNA polyplex micelle alone, viz., the transfection efficiency was found to be 3.2×10^5 (RLU/mg protein) and 4.2×10^6 (RLU/mg protein) for the PEG-*b*-PLL/pDNA polyplex micelle alone and the PEG-*b*-PLL/pDNA polyplex micelle with the lac-nanogel-8k-1.0%, respectively. This almost 13-fold increase in transfection efficiency in lac-nanogel-8k-1.0% is remarkable because other polyplex micelle system in the presence of HCQ (100 μM) showed the 10- and 15-fold enhancement of the transfection efficiency for the HuH-7 cells (same cell line) [19] and HepG2 cells [4], respectively. In addition, significant increase in the transfection efficiency of the PEG-*b*-PLL/pDNA polyplex micelle with nanogel-8k-1.0% was also observed for 293T cells (human kidney; see Fig. S2). These results suggest that after the partial co-internalization of the PEG-*b*-PLL/pDNA polyplex micelle and lac-nanogel-8k-1.0% into the HuH-7 cells, the protonation of the PAMA core of lac-nanogel-8k-1.0% occurred in synchronously with the pH decrease in the endosomal compartment (pH=6∼5), leading to the significant swelling of the PAMA core of lac-nanogel-8k-1.0% (pH=8.0, 84.7 nm → pH=6.0, 160.8 nm in Table 1) to increase the ion osmotic pressure (buffer effect). This may induce the disruption of the endosome, facilitating the transport of the co-internalized PEG-*b*-PLL/pDNA polyplex micelle into the cytoplasm.

To examine whether the lactose moiety (galactose terminal) on the surface of lac-nanogel-8k-1.0% is recognized by the ASGP receptors existing on the HuH-7 cells, a transfection study of the PEG-*b*-PLL/pDNA polyplex micelle with lac-nanogel-8k-1.0% was also performed in the presence of ASF. Note that the ASF is known to strongly interact with ASGP receptors, viz., ASF acts as a competitive inhibitor of the ASGP receptor-mediated endocytosis [24]. A significant decrease in the transfection efficiency of the PEG-*b*-PLL/pDNA polyplex micelle with lac-nanogel-8k-1.0% was observed in the presence of ASF, indicating that the cellular association and internalization of lac-nanogel-8k-1.0% along with the PEG-*b*-PLL/pDNA polyplex micelle occur mainly through the ASGP receptor-mediated process, which is inhibited in the presence of ASF. Thus, it seems reasonable to conclude that an appreciable fraction of the lac-nanogel-8k-1.0% along with the PEG-*b*-PLL/pDNA polyplex micelle is taken up by HuH-7 cells through the ASGP receptor-mediated endocytosis process mediated by the cluster of a large number of lactose moieties on the surface of the lac-nanogel-8k-1.0%, followed by the effective disruption of the endosome by the buffer effect of the unprotonated PAMA core in lac-nanogel-8k-1.0%.

In conclusion, the pH-responsive and targetable PEGylated nanogel (lac-nanogel-8k-1.0%) constructed from a cross-linked polyamine core and tethered PEG chains bearing a lactose molecule exhibited significant endosomolytic ability, achieving the pronounced transfection efficiency of the PEG-*b*-PLL/pDNA polyplex micelles without any cytotoxicity. Therefore, the pH-responsive and targetable PEGylated nanogel thus described in this paper would be a promising targetable and biocompatible endosomolytic agent for nonviral gene delivery systems.

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